Limited Proteolysis of Tubulin: Nucleotide Stabilizes an Active Conformation†

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ABSTRACT: Limited proteolysis has been used to examine tubulin structure as related to microtubule assembly. Purified tubulin, freed of exchangeable nucleotide, was digested with low concentrations of chymotrypsin (0.01–0.1% w/w to tubulin) and its polymerization behavior investigated. Chymotryptic proteolysis resulted in a loss of assembly activity with apparent first-order inactivation kinetics. The inactivation rates were dependent on both chymotrypsin concentration and incubation temperature. However, these conditions of proteolysis did not significantly affect tubulin's colchicine binding activity. Polyacrylamide–$NaDodSO_4$ electrophoresis demonstrated the major cleavage fragments of tubulin to be 34 and 17 kilodaltons. Furthermore, amino-terminal analysis showed methionine for the 17-kilodalton fragment and both glutamate and serine for the 34-kilodalton fragment. Microtubular structures formed from chymotryptic tubulin possessed constrictions and had a frayed appearance in the electron microscope; these polymers were composed of both native tubulin and the 34- and 17-kilodalton fragments, suggesting that the loss of microtubule assembly results from tubulin cleavage and the altered interaction of cleavage fragments with uncleaved tubulin subunits. Interestingly, the readdition of GTP prior to proteolysis significantly protected tubulin's assembly capacity, presumably by stabilizing the fragments in an active conformation as indicated by circular dichroism spectra.

Tubulin assembly into microtubules is one of the most striking systems of protein self-association found in eukaryotic cells. The biological functions of microtubules as well as the chemical characterization of the subunit protein tubulin have been widely investigated (Kirschner, 1978; Raff, 1979; Timasheff & Grisham, 1980). The GTP requirement for microtubule assembly and the role of microtubule associated proteins have also been extensively studied (Maccioni & Seeds, 1977; Carlier & Pantaloni, 1978; Terry & Purich, 1982; Cleveland et al., 1977; Vallee & Borisy, 1977). Although specific amino acid residues have been implicated in microtubule assembly (Lee et al., 1976; Mann et al., 1978; Maccioni et al., 1981; Maccioni & Seeds, 1982a), very little is known about tubulin's structural features involved in the assembly process.

In the present report, limited proteolysis is used as an approach to study the structural domains of tubulin related to its assembly into microtubules. The effect of chymotryptic cleavage of tubulin subunits on microtubule assembly and the protective influence of GTP[1] are examined. A preliminary report of these studies appeared previously (Maccioni & Seeds, 1981).

Materials and Methods

Tubulin from lamb brain was isolated by cycles of assembly–disassembly following the procedure of Shelanski et al. (1973) and stored as a pellet at −70 °C. Immediately before use the pellets were resuspended in 0.1 M Mes (pH 6.8), 0.5 mM EGTA, and 1.5 mM $MgCl_2$ (assembly buffer), a third cycle of assembly was performed, and the tubulin was resuspended in buffer without Mg^{2+}. For most studies (unless otherwise stated) tubulin (PC-tubulin) freed of the residual MAPs by phosphocellulose chromatography (Weingarten et al. (1975) was used. The exchangeable nucleotide and any unbound nucleotide in the tubulin preparation was removed by charcoal extraction in the presence of 1.5 mM EDTA as described previously (Maccioni & Seeds, 1977, 1982b). Charcoal-extracted tubulin contained 1.0 ± 0.1 mol of guanine nucleotide/mol of dimer.

The assembly of PC-tubulin was induced by the addition of purified MAPs or by 10% Me_2SO (Robinson & Engelborghs, 1982) and was monitored by either the turbidimetric (Gaskin et al., 1974) or the sedimentation assay (Johnson & Borisy, 1975). For turbidimetric assays the change in absorbance at 350 nm was recorded during incubation of tubulin solutions at 32 °C in the presence of 1 mM GTP. After reaching a plateau the sample was cooled to 4 °C and the extent of microtubule assembly determined from the cold reversible fraction. Samples incubated in the absence of GTP served as controls for the assembly assay.

The chymotryptic treatment of tubulin samples was performed according to the following steps: tubulin in 0.1 M Mes buffer (pH 6.8), 1.5 mM $MgCl_2$, and 1 mM EGTA was freed of exchangeable and remaining nucleotide by charcoal extraction at 4 °C; samples of this tubulin preparation were incubated with α-chymotrypsin (Sigma, TLCK-treated type VII) at the indicated concentrations and at either 15 or 30 °C; aliquots of the incubation medium were taken at different time intervals, diluted appropriately in assembly buffer containing 0.5 mM PMSF, and assayed for assembly activity. Control samples were incubated under identical conditions but without chymotrypsin. Protein concentration was determined by the procedure of Lowry et al. (1951) or by using a value of 1.15 A_{280}/mg of tubulin (Appu Rao et al., 1978).

Analytical electrophoresis in the polyacrylamide–$NaDodSO_4$ system of Laemmli (1970) was used to determine the effects of chymotryptic proteolysis on the tubulin heterodimer. Aliquots of both chymotryptic-treated and control samples processed under the experimental conditions were immediately boiled in the presence of sample buffer containing 0.5 mM PMSF to avoid further proteolysis. The appearance of low

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; PC-tubulin, phosphocellulose-purified tubulin; Me_2SO, dimethyl sulfoxide; CD, circular dichroism.

0006-2960/83/0422-1567$01.50/0

molecular weight peptides was further analyzed in a 15% polyacrylamide–6 M urea–0.1% $NaDodSO_4$ system with 0.1 M sodium phosphate (pH 7.2). Standard polypeptides used to determine the molecular weights of cleavage products of tubulin were bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 000), cytochrome *c* (12 300), bovine trypsin inhibitor (6200), and insulin peptide chains (3000).

Determination of the amino-terminal residues of the two major polypeptides produced by tubulin cleavage was performed according to the procedure of Hartley (1970). The tubulin cleavage fragments were separated by preparative electrophoresis in a 9% polyacrylamide–$NaDodSO_4$ system. Bands containing the major peptides were excised and the polypeptides eluted in a 0.1 M Mes (pH 6.8) buffer containing 0.1% $NaDodSO_4$ and desalted on a Sephadex G-25 column. An aliquot of each peptide was analyzed for purity in the analytical electrophoresis system (Laemmli, 1970). Briefly, the procedure of Hartley (1970) involves the reaction of the polypeptides with dansyl chloride, hydrolysis with 6 N HCl, and two-dimensional chromatography on polyamide thin-layer plates. A complete set of dansylated amino acids were used as standards to identify the amino-terminal residue of these major polypeptides.

Colchicine binding of digested and undigested PC-tubulin samples were assayed according to Weisenberg et al. (1968). Tubulin samples (50 μL) were incubated at 37 °C for 45 min with 2.4×10^{-5} M [^{3}H]colchicine (sp act. 4.3×10^5 cpm/nmol). After incubation, samples were filtered through two Whatman DE-81 filters and washed 3 times with 0.01 M Mes (pH 6.8) containing 2.4×10^{-5} M colchicine and 5 mM $MgCl_2$, and the radioactivity was determined in a Searle liquid scintillation counter.

Circular dichroism spectra were recorded at 27 °C from 280 to 200 nm by using a Cary Model 60 spectropolarimeter with a unit 6001 CD attachment. The slits were 15 Å at each wavelength and the mean residue ellipticities $(\theta)_{mrw}$ (deg cm^2/dmol) were calculated by using a value of 115 for the mean residue weight.

Results

In order to ascertain the structural features of tubulin related to the assembly process, studies were undertaken on the effects of limited proteolysis of tubulin on its assembly behavior. Microtubule protein purified by three cycles of assembly–disassembly and freed of the exchangeable nucleotide was treated with chymotrypsin, the samples were diluted 10-fold with assembly buffer containing 0.5 mM PMSF, and the assembly activity was examined by both turbidity and sedimentation assays. Tubulin proteolysis at 30 °C resulted in a time-dependent inactivation of the assembly over a wide range of chymotrypsin concentrations (Figure 1). The inactivation of the assembly followed pseudo-first-order kinetics, and a brief lag period was observed at the lower concentrations of the proteolytic enzyme (0.006–0.018% w/w to tubulin). The apparent first-order rate constants for the assembly decay (k_0') were proportional to the increase in chymotrypsin concentration (Figure 1, insert).

An inactivation was also observed when phosphocellulose-purified tubulin (PC-tubulin) was treated with chymotrypsin and induced to assemble following the addition of MAPs. The effects of the chymotryptic digestion of PC-tubulin were analyzed in the presence and the absence of GTP. Figure 2 shows the time-dependent inactivation of PC-tubulin assembly by proteolysis at 15 °C with 0.036% w/w chymotrypsin. This and all the following studies were performed at 15 °C to

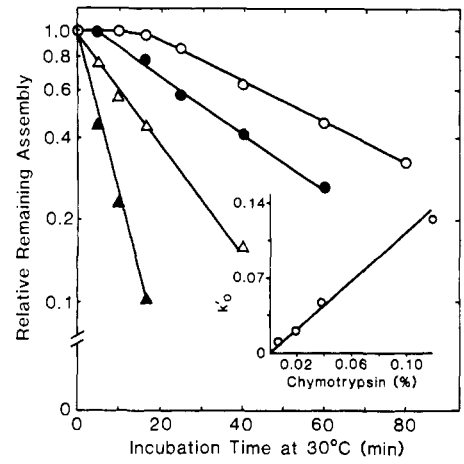

FIGURE 1: Time-dependent inactivation of tubulin assembly by proteolysis at different concentrations of chymotrypsin. Charcoal-extracted microtubule protein samples (cycled 3 times) at a final concentration of 10 mg/mL were incubated at 30 °C in the presence of chymotrypsin at the following concentrations (w/w to tubulin): 0.006% (○); 0.018% (●); 0.036% (△); 0.12% (▲). Aliquots were withdrawn at the time intervals indicated, diluted 10-fold in assembly buffer containing 0.5 mM PMSF, and assayed for assembly by the turbidity method. Assembly activity is represented as a fraction of the assembly extent of control (no proteolysis) at time zero. The apparent first-order inactivation rate constants (k_0') are plotted against the concentration of chymotrypsin (insert).

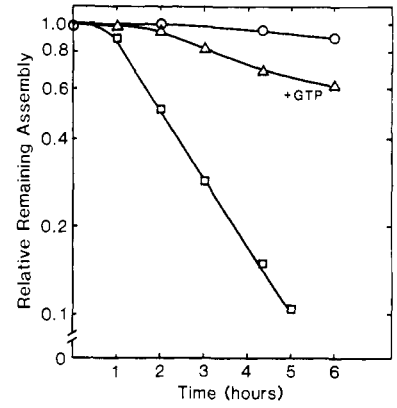

FIGURE 2: Effect of GTP on the tubulin inactivation by limited chymotryptic proteolysis. A 1-mL PC-tubulin sample (10 mg/mL) was incubated at 15 °C with chymotrypsin (0.036% w/w to tubulin), and aliquots were taken at the indicated time intervals, diluted appropriately, and assayed for assembly by using the turbidity method (□). Another sample was preincubated at 4 °C for 15 min with 2 mM GTP and 2.5 mM $MgCl_2$ prior to chymotrypsin addition (△). A control sample was incubated in the absence of both GTP and chymotrypsin, and aliquots were assayed for assembly as indicated (○).

prevent aggregate formation or GTP-induced assembly during digestion. Inactivation followed biphasical kinetics with a lag period for both GTP-protected (~90 min) and unprotected tubulin (~45 min), while control samples (without enzyme) showed only a slight decrease in the assembly activity over the 6 h. Clearly, preincubation of tubulin with 2 mM GTP significantly diminished the inactivation as compared with that of the unprotected sample. Similar results were obtained by using the cycled microtubular protein preparation. The effects of chymotryptic treatment on PC-tubulin were also assessed by the sedimentation assay with samples induced to assemble in the presence of 10% Me_2SO (without MAPs). Table I shows the loss of assembly activity of PC-tubulin digested in the presence and in the absence of GTP, at two different times of digestion. The protection afforded by GTP corresponds well to the values obtained by turbidity measurement.

Table I: Sedimentation Analysis of Tubulin Assembly following Digestion with Chymotrypsin[a]

digestion conditions	incubation time (min)	microtubules (μg)	relative assembly
control	150	340	1.00
(no enzyme)	300	323	0.95
chymotrypsin	150	137	0.40
	300	28	0.08
chymotrypsin	150	299	0.88
+ GTP[b]	300	208	0.61

[a] PC-tubulin samples (12.2 mg/mL) were incubated at 15 °C in the absence (control) or the presence of chymotrypsin (0.036% w/w). Aliquots of 80 μL obtained at the time intervals indicated were diluted 1:5 with assembly buffer containing 0.5 mM PMSF, induced to assemble in the presence of 10% Me_2SO, and assayed by the sedimentation method. [b] Preincubated 10 min at 4 °C with 2 mM GTP prior to digestion.

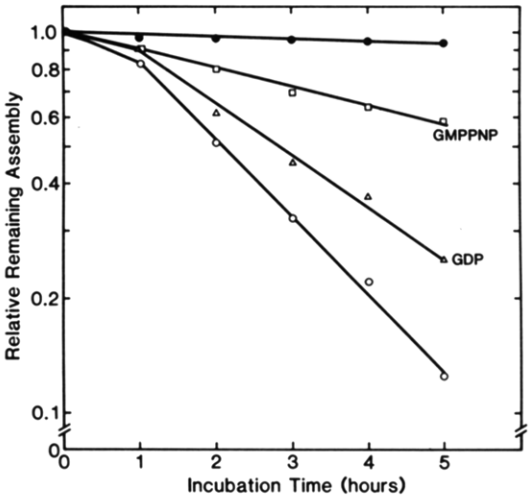

FIGURE 3: Effects of GMPPNP and GDP on tubulin inactivation by chymotryptic proteolysis. A 1-mL PC-tubulin sample (10 mg/mL) was incubated at 15 °C with chymotrypsin (0.036% w/w to tubulin), and aliquots were withdrawn at times indicated, diluted appropriately, and assayed for assembly (○). Other tubulin samples were preincubated at 4 °C for 15 min, either with 2 mM GMPPNP (□) or with 2 mM GDP (△) treated with chymotrypsin, and aliquots were assayed for assembly. A control sample incubated in the absence of both chymotrypsin and the nucleotides was assayed under the same conditions (●). The same results were obtained by using the sedimentation assay for monitoring tubulin assembly.

The chymotryptic digestion in the presence of the GTP analogue GMPPNP or GDP was also analyzed. The protective effect provided by GMPPNP was nearly the same as that seen with GTP; however, only a slight decrease in the inactivation rate as compared with that of the unprotected sample was observed when digestion was performed in the presence of GDP (Figure 3).

Colchicine binding activity of chymotrypsin-treated tubulin was also examined (Table II). The colchicine binding activity is presented as the ratio of the experimental sample to a control sample, which compensates for the spontaneous decay of colchicine binding activity during the incubation period (Bamburg et al., 1973). Colchicine binding activity of tubulin did not change significantly after proteolysis over a wide range of chymotrypsin concentrations. Under conditions where virtually all (>90%) the assembly capacity was lost, i.e., 0.16% chymotrypsin, colchicine binding was decreased only 8%.

The structural consequences of tubulin digestion were analyzed by electrophoresis. Figure 4a shows a polyacrylamide–$NaDodSO_4$ slab gel of tubulin samples digested for 20 and 260 min with 0.036% w/w chymotrypsin, in the absence

Table II: Colchicine Binding of Tubulin Treated with Increasing Chymotrypsin Concentrations[a]

chymotrypsin (%)	mol of [^{3}H]colchicine/ mol of tubulin	ν_d/ν_c[b]
0	0.48	1.00
0.02	0.48	1.00
0.04	0.45	0.94
0.08	0.46	0.95
0.16	0.44	0.92
0.30	0.44	0.92
0.48	0.41	0.85
0.08[c]	0.47	0.98
0.48[c]	0.43	0.89

[a] Tubulin samples (10 mg/mL) were incubated at 15 °C for 260 min with chymotrypsin at the concentrations indicated. Aliquots of 5 μL were diluted 10-fold in assembly buffer with 0.5 mM PMSF and assayed for colchicine binding in the presence of 2.4×10^{-5} M [^{3}H]colchicine as indicated under Materials and Methods. [b] ν_d: colchicine binding of chymotryptic-treated samples. ν_c: colchicine binding of the control. [c] Digestion in the presence of 2 mM GTP.

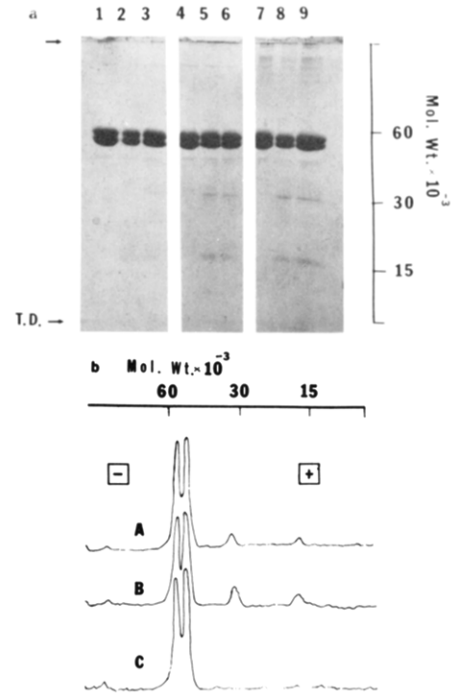

FIGURE 4: Polyacrylamide–$NaDodSO_4$ slab gel of PC-tubulin digested for different time intervals. (a) Samples of PC-tubulin (9 mg/mL) were incubated at 15 °C with or without chymotrypsin, aliquots of 8 μL were obtained at 20 min (lanes 1, 2, and 3) and 260 min (lanes 4, 5, and 6), the digestion was terminated by boiling samples with 1% $NaDodSO_4$, 3% 2-mercaptoethanol, 1 M glycerol, and 0.5 mM PMSF, and the samples were electrophoresed in 9% acrylamide gels according to Laemmli (1970). Control samples incubated without chymotrypsin (lanes 1, 4, and 7) and samples incubated with chymotrypsin (0.036% w/w) either in the absence (lanes 2, 5, and 8) or in the presence of 2 mM GTP (lanes 3, 6, and 9). Similarly treated samples of PC-tubulin (7, 8, and 9) were induced to assemble by 10-fold dilution into assembly buffer containing 2 mM GTP and exogenous MAPs (0.65 mg/mL) at 37 °C. The polymerized tubulin was collected by centrifugation and resuspended, and 70 μg of protein was processed for electrophoresis as described above. (b) Densitometric tracing of PC-tubulin samples (lanes 6, 5, and 4, respectively) incubated 260 min with chymotrypsin in the presence (A) or the absence (B) of GTP and the undigested tubulin control (C).

(lanes 2 and 5) and the presence of GTP (lanes 3 and 6), and samples incubated without chymotrypsin (control, lanes 1 and 4). The last three lanes (7–9) are samples of microtubule pellets obtained from the assembly of digested PC-tubulin and

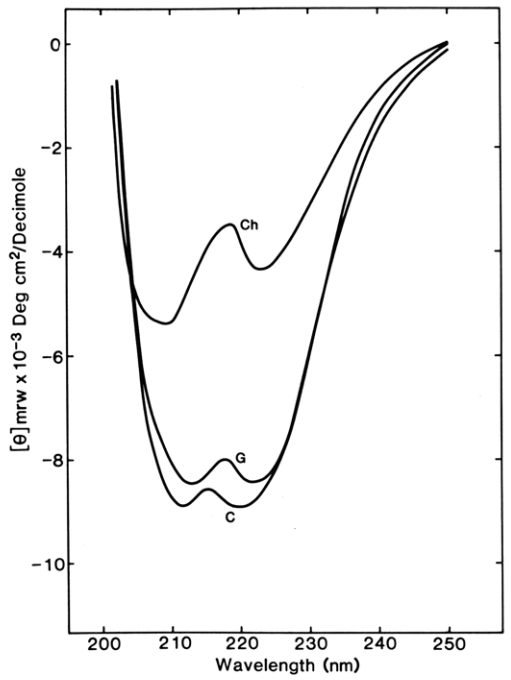

FIGURE 5: Far-ultraviolet CD spectra of PC-tubulin incubated in the presence or the absence of chymotrypsin. Charcoal-extracted PC-tubulin samples (9 mg/mL) were incubated at 15 °C for 260 min with chymotrypsin (0.05% w/w) either in the absence (Ch) or in the presence (G) of 2 mM GTP. A control sample (C) was incubated in the presence of 2 mM GTP but without chymotrypsin. Samples were passed through Sephadex G-25 columns, equilibrated in 0.01 M Mes–1.5 mM $MgCl_2$ to a final protein concentration of 0.32 mg/mL, and analyzed by circular dichroism. Another control sample which was incubated in the absence of GTP and chymotrypsin showed the same CD spectra as that of C. Other experimental conditions are described under Materials and Methods.

exogenous undigested MAPs. This electrophoretic analysis shows that the major products of digestion were polypeptides of 34 and 17 kilodaltons. Figure 4b represents a densitometric tracing of the same gel of PC-tubulin samples incubated 260 min with chymotrypsin in the presence and the absence of GTP and the undigested tubulin control (lanes 4, 5, and 6). Although microtubule assembly was inhibited by approximately 85% in the unprotected sample, less than 17% of the total tubulin was cleaved to smaller size species during proteolysis (lane B). Furthermore, the cleaved fragments are incorporated into MAP-induced polymers (lanes 8 and 9) in an amount similar to their abundance in the digested tubulin samples (lanes 5 and 6).

The extent of cleavage increased with chymotrypsin concentration (from 0.004% to 0.43% w/w) with the 34- and 17-kilodalton peptides still representing the major components, although some minor components in the region of 32–38 kilodaltons were often observed and a small amount of α-tubulin remained when all the β-tubulin had been digested (data not shown). In addition, a partial protective effect by GTP of tubulin proteolysis was suggested by these studies (compare Figure 4b, lanes A and B). Therefore, as a control, the chymotryptic proteolysis of a non-GTP binding protein, ovalbumin, was compared under similar conditions in the presence and absence of GTP. There were no differences in the digestion products, thus ruling out inhibition by a direct interaction between GTP and chymotrypsin.

Following their isolation from polyacrylamide gels, the 34- and 17-kilodalton peptides were reacted with dansyl chloride to identify their amino-terminal residues. Two-dimensional chromatography of the acid hydrolysates (Hartley, 1970) showed only methionine as the N-terminal amino acid of the

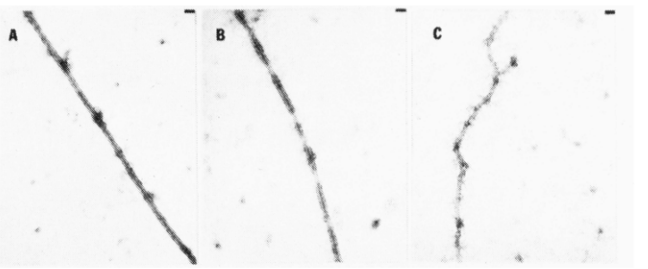

FIGURE 6: Electron microscopy of the assembly products of untreated and chymotryptic-treated tubulin. (A) Phosphocellulose-purified tubulin was incubated at 15 °C for 260 min and then diluted into assembly buffer containing untreated MAPs and 1 mM GTP at 37 °C, and aliquots were prepared for electron microscopy as indicated under Materials and Methods. Magnification bar equals 500 Å. (B) Tubulin sample incubated with 0.036% w/w chymotrypsin in the presence of 2 mM GTP and assembled as in (A). (C) Tubulin sample incubated with chymotrypsin in the absence of the nucleotide and assembled as in (A).

17-kilodalton fragments and both glutamate and serine as N-terminal residues for the 34-kilodalton fragments.

The ability of GTP to protect tubulin's assembly activity even though tubulin was similarly cleaved by chymotrypsin suggested that a conformational difference may exist between chymotryptic tubulin samples incubated in the presence and absence of nucleotide. Circular dichroism spectra (Figure 5) showed that there was no significant difference in the conformation of tubulin treated with chymotrypsin in the presence of GTP (G) as compared to that of the undigested control (C); however, a substantial conformational change resulted from digestion under nonprotective (minus GTP) conditions (Ch). In this and previous studies (Maccioni & Seeds, 1982b) we have shown that there is no significant difference in the CD spectra of nondigested tubulin samples when examined in the presence or absence of nucleotide. Furthermore, analysis of these CD spectra according to the procedure of Greenfield & Fasman (1969) indicated 12% α helix, 45% β-pleated sheet, and 43% random coil for the control (untreated) tubulin and after chymotryptic digestion in the presence of GTP 10% α helix, 46% β-pleated sheet, and 44% random coil; however, after digestion in the absence of nucleotide α helix was decreased to 0.3% while β-pleated sheet was 43% and random coil increased to 56.7%.

Electron microscopy of the assembly products of PC-tubulin revealed a decrease in the number and length of tubules formed after increasing the time of exposure to the proteolytic enzyme. Figure 6 shows microtubules assembled from tubulin incubated at 15 °C without chymotrypsin (control A) and the assembly products from tubulin digested for 260 min in the presence of GTP (B) or in the absence of nucleotide (C). The protective effect of GTP was evidenced again in these electron microscope observations of microtubules. In general, shorter and constricted microtubules were observed in the GTP-protected sample, compared to the control, while incomplete tubules and filamentous structures were the most abundant products of assembly in the unprotected digest sample.

Discussion

These studies have shown that one site in the tubulin monomer is particularly sensitive to proteolytic cleavage by chymotrypsin at concentrations as low as 0.01% (w/w), producing 17- and 34-kilodalton fragments; furthermore, cleavage in the absence of exchangeable nucleotide led to a rapid loss of tubulin's ability to form microtubules. Microtubule assembly was inhibited to the same extent when either exogenous phosphocellulose-purified MAPs or 10% Me_2SO (Robinson

& Engleborghs, 1982) was used to induce assembly of chymotryptic tubulin. In control studies the simultaneous addition of chymotrypsin, PMSF, and purified MAPs to a tubulin solution did not produce inactivation, nor was there detectable cleavage of the microtubule associated proteins under the experimental conditions used in this study. In addition, both phosphocellulose-purified tubulin and DEAE-Sephadex-purified tubulin (Eipper, 1975) (data not shown) displayed similar inactivation kinetics and cleavage products, indicating the loss of assembly was directly related to the proteolytic cleavage of tubulin.

Amino-terminal analysis of tubulin and the cleavage fragments indicated that methionine was the N terminal for both tubulin and the 17-kilodalton fragment, while glutamate and serine were both found as amino-terminal residues in the 34-kilodalton fragments. These findings suggest that chymotrypsin preferentially cleaves α- and β-tubulin to generate 17-kilodalton fragments that represent the amino-terminal one-third of each monomer. The identification of both glutamate and serine as the amino-terminal residues in the 34-kilodalton fragment coupled with the substrate specificity for chymotrypsin and the published sequence data for chick (Valenzuela et al., 1981) and pig brain tubulin (Krauhs et al., 1981) suggests that the cleavage site is between Phe_{135} and Ser_{136} in α-tubulin and Phe_{133} and Glu_{134} in β-tubulin. The conserved homology between chick and pig brain tubulin sequences sugests that lamb brain would possess a similar sequence. It is interesting to note that the α-subunit sequence shows several potential cleavage points in this region at Phe_{138}–His_{139}, Phe_{141}–Gly_{142}, and Phe_{149}–Thr_{150}, which may explain the presence of some minor components between 32–36 kilodaltons occassionally observed in $NaDodSO_4$–polyacrylamide gel electrophoresis. In addition, at high chymotrypsin concentrations or long incubation times a small amount of α-tubulin could still be seen when all the β-tubulin had disappeared from the $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown), suggesting the β subunit may be more readily susceptible to cleavage.

The observation that cleavage of only 17% of the tubulin resulted in an 85% inhibition of assembly was surprising. However, $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the tubulin polymers formed with chymotryptic tubulin indicated that the two cleavage fragments bound to the growing polymer as well as uncleaved tubulin (Figure 4). These findings suggest that the bound fragments prevent normal association of additional tubulin subunits and lead to the aberrant filamentous structures seen in Figure 6. This idea is in agreement with the EM observations of progressively shorter tubules and increased filamentous forms as digestion time increased.

Earlier studies by Vallee & Borisy (1977) showed that high molecular weight MAPs were very sensitive to trypsin. Incubation with intact microtubules led to the loss of lateral projections and cleavage of high molecular weight MAPs into several components including a 35-kilodalton peptide that was closely associated with tubulin; however, the tryptic-treated protein could reassemble. Using a high trypsin concentration Love et al. (1981) found a 41-kilodalton peptide to be the major cleavage product of both intact microtubules and cold depolymerized microtubular protein (MAPs and tubulin). In a recent report Brown & Erickson (1981) confirmed the 41-kilodalton peptide from the tryptic digest, as well as finding 34- and 19-kilodalton peptides from chymotryptic-treated tubulin; however, there was no chymotryptic cleavage of assembled microtubules. Interestingly, their chymotryptic tubulin was capable of reassembly; however, these preparations had not been freed of exchangeable and unbound nucleotide prior to digestion.

Although chymotryptic cleavage inhibited tubulin assembly into microtubules and had a marked impact on tubulin structure, particularly its α-helix content, as indicated by CD spectra (Figure 5), proteolysis had very little effect on tubulin's ability to bind colchicine (Table II). The relative insensitivity of colchicine binding suggests that the binding site for this alkaloid drug is probably quite distant from the chymotryptic cleavage site.

The most striking feature of the limited proteolysis of tubulin was the protective effect of GTP and the analogue GMPPNP against loss of activity by proteolysis. The possibility that protection exerted by the nucleotide could be a consequence of microtubule formation was ruled out by performing the chymotryptic treatment at 15 °C, where tubulin does not assemble. Circular dichroism spectra (Figure 5) indicate that guanine nucleotide stabilized tubulin in an active conformation required for assembly; however, $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4) showed that tubulin was cleaved into 17- and 34-kilodalton fragments in the presence and absence of GTP. Thus, the interaction of GTP with tubulin either directly or indirectly promotes the noncovalent association of these two fragments. Although the noncovalent forces between nucleotide and the two protein fragments may not be sufficient to stabilize the molecule, they may promote additional protein–protein interactions that can effectively stabilize the structure; alternatively, GTP bound at a distant site may allow the proper protein–protein interaction between the cleaved fragments. A direct role of GTP in the stabilization process is suggested by the observation (Maccioni & Seeds, 1983) that the GTP binding site is very near the proposed chymotryptic cleavage site, such that GTP may bridge the two fragments. However, these studies also demonstrate that GTP does not promote the reassociation of cleaved fragments into an active conformation, since the presence of GTP in the assembly buffer is unable to induce microtubule formation.

The minimal protection provided by GDP may be related to GDP–tubulin's relatively inactive role in microtubule assembly and that GDP–tubulin possesses a sightly different conformation than its GTP counterpart as suggested by Karr & Purich (1978) from fluorescence quenching studies, thus preventing an effective stabilization of the two fragments.

These studies have indicated the potential of limited proteolysis as a dissecting tool in tubulin structure, and future studies with other proteases and experimental conditions should produce additional information on specific structural and functional domains of the tubulin molecule.

Acknowledgments

We are grateful to Dr. J. R. Cann and R. Coombs for their assistance with the circular dichroism studies.

Registry No. GTP, 86-01-1; GMPPNP, 34273-04-6; GDP, 146-91-8.

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Affinity Labeling of Tubulin's Exchangeable Guanosine 5′-Triphosphate Binding Site†

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ABSTRACT: Tubulin requires GTP for maximal rate and extent of polymerization into microtubules. The localization of the guanine nucleotide in the microtubule was examined by preparing affinity probes that would permit tubulin polymerization prior to their covalent coupling to amino acids in tubulin's exchangeable GTP binding site. Two different hydrolyzable GTP analogues with modified ribose moieties, 3′-*p*-azidobenzoyl)-GTP and the periodate oxidation product of GTP, 2-(guanylformylmethoxy)-3-(triphospho)propanal, were isolated by thin-layer chromatography and high-voltage electrophoresis and identified by ultraviolet and infrared spectroscopy. The analogues bind to the tubulin molecule and promote polymerization. After tubulin polymerization and isolation of microtubules, the [^{3}H]GTP analogues were covalently coupled to tubulin by $NaBH_4$ reduction or UV irradiation. The microtubules possessed about 1 mol of acid-precipitable ^{3}H-labeled nucleotide/mol of tubulin dimer. Separation of the subunits showed that the nucleotide analogues were associated with both α and β subunits of tubulin in nearly equal amounts. The binding of analogues to both α and β subunits was saturable and competitive with GTP. Cyanogen bromide cleavage of both α and β subunits showed that the ^{3}H-labeled nucleotide was associated with a single molecular weight species of similar size (~10000) from each subunit. Two-dimensional electrophoresis of chymotryptic peptides from both (α and β) cyanogen bromide fragments showed that the ^{3}H-labeled nucleotide was associated with a peptide of nearly identical migration properties from both subunits. These results suggest that a similar peptide segment of both α- and β-tubulin has the ability to bind GTP. Furthermore, this peptide was localized to the amino-terminal one-third of the tubulin molecule.

Microtubules are involved in a variety of cellular functions (Roberts & Hyams, 1979). Assembly of microtubules from the subunit protein tubulin, a M_r 110000 heterodimer of α and β monomers, requires guanosine triphosphate (GTP)[1] (Borisy & Olmsted, 1972; Maccioni & Seeds, 1977a). The tubulin dimer has two guanine nucleotide binding sites of

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GTPox, the periodate oxidation product of GTP; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PEI–cellulose, poly(ethylenimine)–cellulose; PMSF, phenylmethanesulfonyl flouride.

0006-2960/83/0422-1572$01.50/0